Award Number: W81XWH-10-2-0120

TITLE: Evaluation of the Human/Extreme Environment Interaction: Implications for Enhancing Operational Performance and Recovery

PRINCIPAL INVESTIGATOR: Brent C. Ruby, Ph.D., FACSM

CONTRACTING ORGANIZATION: The University of Montana
Missoula, MT, 59812

REPORT DATE: October 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
30 October 2012	Annual	1 Oct 2011- 30 September 2012

4. TITLE AND SUBTITLE
Evaluation of the Human/Extreme Environment Interaction: Implications for Implications for Enhancing Operational Performance and Recovery

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-2-0120

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Brent C. Ruby, Ph.D.

E-Mail: brent.ruby@mso.umt.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Montana
Missoula, MT 59812

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this research determines how hypoxia interacts with exercise and recovery to yield various metabolic responses that may affect performance and performance at high-altitude critical to mission success. Participants (n=10) completed two exercise trials under normoxic conditions (975 m) that included a 90-minute interval protocol (cycling). Subjects then recovered for 4 hours under the same normoxic conditions or at a high, simulated altitude of 5000 m. Muscle biopsies from the vastus lateralis were obtained before exercise, after exercise and 6 hours after exercise for the measurement of metabolic gene expression and muscle glycogen. Muscle glycogen decreased with exercise and increased with recovery with no difference between trials. The majority of the metabolic genes were suppressed after altitude exposure. These data demonstrate that high altitude exposure during recovery from exercise inhibits gene expression associated with mitochondrial development without affecting the recovery of muscle fuels.

15. SUBJECT TERMS- hypoxia, exercise recovery, oxidative stress, metabolic genes, mitochondrial genes

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	36	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

INTRODUCTION:

Highly trained military forces must perform at peak performance during combat operations. The main goals of this proposal have been to measure changes in muscle cells after exercise in cold and high altitude environments. Because the stresses of hard work can affect how well a soldier may perform on the next mission/assignment, we are interested in how the environmental stress and selected nutrition may interact during recovery. This research plan uses methods that have direct application to the soldier's operational environment. The altitude stress included has direct relevance to the current conflicts and U.S. troop presence in Afghanistan.

These studies have been developed to determine the effects of harsh environments on the body and what types of pre-mission training and what types of mission nutrition can improve performance, safety and recovery of the soldier. The application of these results should be used during early training of soldiers to increase the muscles adaptation(s) to training. During this second year of the project series our specific aim was to determine the impact of exercise and recovery under hypoxic stress on metabolic genes and oxidative stress markers. These data build of our findings from year 1 by advancing our understanding of how acute the exercise stress may interact with environmental conditions to alter the responsiveness of the skeletal muscle. These data have further implications on training and environmental exposure strategies and how they could be used to enhance mission preparedness.

BODY:
The central scientific question from this research revolves around how hypoxia interacts with exercise and recovery to yield various metabolic adaptations that may affect performance and performance at high-altitudes specific to the operational environment. The process or our work has been outlined below relative to our original statement of work aligned with year 2. Throughout the project, we have remained on schedule or slightly ahead of schedule and are currently in the process of finalizing our IRB/HRPO approval for the first segment of year 3.

Year 2
Task 5. Data collection for lab study 2 (months 12-16).

5a. Revise approved IRB protocols for any necessary changes suggested by laboratory study 1 and recruit subjects (months 12-13).

Based on our discussions regarding minor adjustments to the experimental conditions, we completed the second IRB application, which was reviewed in late August, 2011. This application was completed immediately after our research team discussions and was approved by the University in late August 2011. Following University IRB approval, the application was submitted on August 31 by our Center to HRPO at Fort Detrick. Because of our initial work with Sharon Evans on our first IRB submission (lab study 1), all our paperwork was compiled and submitted quickly. HRPO approval was granted on September 22, 2011 (a new record for our research Center as we typically anticipate a 2-3 month review and approval process).

This has put us ahead of schedule and has allowed us to coordinate some maintenance to our chamber and to better prepare us for an early start to the data collection period for the next laboratory study. We will be initiating pilot testing for this phase of the project by mid November.

Task 5. Data collection for lab study 2 (months 12-16).

5a. Revise approved IRB protocols for any necessary changes suggested by laboratory study 1 and recruit subjects (months 12-13).
5b. Data collection for laboratory study 2 (months 14-16).

This was completed in October, November and December of 2012.

Task 6. Data analyses for laboratory study 2 (months 15-20).
6a. Order necessary analytical kits, probes, primers and other analyses needs (month 16).
6b. Finalize analytical techniques and analyze all samples (months 16-18).
6c. Data analyses of descriptive and other data collected (non biochemical) (months 18-19).
6d. Statistical analyses of all data from study 2 (months 19-20).

These steps have been completed according to the original timeline. Sample analyses was extensive and has just recently been completed for both the large number of muscle and

blood analytes. The analyses of all the descriptive data measured for each trial of the study has been completed.

During the period of August and September we developed the necessary protocol changes for laboratory study three.

Task 7. Data collection for lab study 3 (months 19-22).

7a. Revise approved IRB protocols for any necessary changes suggested by laboratory study 1 and 2 and recruit subjects (month 19).

We have recently obtained IRB approval from the University and are currently preparing the necessary documentation to submit for HRBO review. We anticipate that this submission will occur on or before November 2, 2012.

Findings from year 2

INTRODUCTION

It has been largely assumed since 1962 that hypoxic conditions and/or altitude stimulate muscle oxidative capacity. This notion comes from the observation that active Peruvian miners had 78% more cytochrome c reductase and 16% more myoglobin than low land controls (34). Five years later it was noted that endurance exercise was a potent stimulator of mitochondrial enzymes (16). The data from these two studies formed the consensus that muscle hypoxia (from exercise or environment) was an important stimulus for mitochondrial development. In the early 1990's this tenant was challenged by data before and after mountaineer expeditions to the Himalayas. The findings of these studies demonstrated a loss of muscle cross sectional area, decreased mitochondrial volume, and decreased maximal aerobic capacity (9, 18, 19). Additionally, after return from these expeditions lipofuscin, a mitochondrial breakdown by-product, was increased (28). These findings of reduced oxidative capacity were further confirmed by "Expedition Everest II" (27). Low levels of mitochondrial capacity have also been shown in the high altitude residents of La Paz (7) and in Sherpa's (22). Paradoxically, these high land populations have excellent physical performance at altitude.

When exercise training under hypoxic conditions, but recovering in a normoxic environment, a slightly different story emerges (train high, live low). When this type of short-term exposure is incorporated into a training paradigm mitochondrial density, maximum aerobic capacity, citrate synthase activity, and anaerobic performance is enhanced over normoxic control exercise (4, 6, 11, 14, 29, 30, 39, 40). Functionally, increased glucose metabolism shown with altitude exposure and acclimatization (5) may be more efficient in that this adaptation would lead to more ATP per molecule of oxygen. The paradox with short-term and long-term metabolic adaptations is yet to be completely understood, however it may be related to the recovery process and specifically indications of decreased protein synthesis during hypoxia (13).

The mechanisms to which hypoxic adaptations occur are even less clear, but seem to be regulated by hypoxia-inducible factor 1 (HIF-1) (36). HIF-1 is a transcription factor that

stabilizes in the nucleus upon exposure to hypoxic conditions (20, 38) and in turn induces the expression of hypoxia-induced genes. HIF-1 causes increased gene expression for glucose transporters, glycolytic enzymes, angiogenic factor, and erythropoietin by targeting the hypoxic response element in the promoter region (33, 36). Higher HIF-1 mRNA levels after training (40, 43) may produce an increased potential for quick activation of HIF-1 with the onset of hypoxic stimuli after training at altitude (17). However, the response of HIF-1 after exercise training is blunted in response to an absolute intensity exercise protocol l(26). Thus, HIF-1 stabilization may be increased by exercise and factors independent of hypoxia. Indeed, interleukin-1beta, insulin like growth factor I and II, insulin, heregulin, epidermal growth factor TNF alpha, angiotensin-2, and nitric oxide have been shown to be capable of inducing HIF-1 (8, 12, 15, 24, 35, 42). The complex nature of HIF-1 activation serves a common endpoint to deliver oxygen and up-regulate the metabolic machinery of the cell.

Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) gene expression is strongly induced by exercise (1, 23, 32) contributing to mitochondrial biogenesis and metabolic alterations. Recent data from our lab has shown PGC-1α mRNA to increase with acute exercise, but have a blunted response after 11 and 21 days of endurance training (37). PGC-1α expression is also induced by hypoxia (41) and does not appear to be dependant on HIF-1. The activation of PGC-1α stimulates mitochondrial biogenesis and hence contributes to increased fatty acid fuel oxidation and subsequent exercise performance (25, 41).

The current view of the mitochondria is not one of many mitochondria, but of a single mitochondrial complex within a cell where the solitary shapes of mitochondria are considered to be sections of mitochondrial tubules. Fusion (growth and development) of these tubules has been shown in healthy conditions such as exercise and weight loss (10, 31) while fission (degradation, dysfunction) is associated with obesity and disease (2, 3). We have recently observed an increase in Mitofusin 2 (MFN2) mRNA, a mitochondrial fusion gene with acute exercise throughout 21 days of endurance training (37). It has been suggested that the balance between fusion and fission may play a central role in the metabolic consequences of hypoxia(21).

These two distinct pathways of metabolic alteration with hypoxia (1. HIF-1 and 2. PGC-1α) have very different effects. The HIF-1 pathway leads to increased reliance on carbohydrate substrates and the PGC-1α pathway leads to increased reliance on fat substrates. Clearly, investigations are needed to discern the pathways and applied metabolic outcomes associated with hypoxia. This information will allow for protocol development for not only altitude/hypoxia tolerance, but also for exercise performance, both of which may be critical to mission success in harsh environments.

This investigation aimed to determine metabolic, morphologic, and oxidative stress gene expression related to mitochondrial development after exposure to simulated high altitude (5000 m). The novel use of this very high altitude will allow insight into mitochondrial response using a very robust hypoxic stimulus. These data have implications into the efficacy of training at such a high altitude in order to stimulate enhanced exercise performance and thus increased probability of mission success.

METHODS

Participants

Twelve male participants (25 ± 2 yrs, 178 ± 7 cm, 79 ± 8 kg, 4.2 ± 0.6 L•min^{-1} completed the study. Subjects completed a Physical Activity Readiness Questionnaire (PAR-Q) and were briefed on the experimental protocol and possible risks prior to giving written informed consent. All procedures were approved by the University Institutional Review Board (The University of Montana, Missoula, MT).

Preliminary Testing

Body composition was measured using hydrodensitometry. Underwater mass was measured with a digital scale (Exertech, Dreshbach, MN). Body density was corrected for estimated residual lung volume (3) and converted to percent body fat using the Siri equation (9). A graded maximal exercise test (starting at 95 W, and increasing 35 W every 3 minutes) was completed on an electronically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA) to determine maximal aerobic capacity (VO_{2max}) and the power output associated with VO_2max (W_{max}). Expired gases were measured during the test, using a calibrated metabolic cart (ParvoMedics, Inc., Salt Lake City, UT). VO_{2max} was assigned to the highest achieved oxygen uptake recorded during the test. W_{max} was calculated by adding the power output in the last completed stage to the fraction of time spent in the uncompleted stage multiplied by 35.

Experimental Protocol

Design.

Participants completed 2 trials by cycling for 90 minutes in laboratory conditions and recovering in 2 different hypoxic environments [975 meters and 5000 meters] using a randomized, counterbalanced cross-over design over the span of 2 weeks, with a minimum of 7 days between trials. All trials were completed in a temperature, humidity, and hypoxia (Colorado Altitude Training, Louisville, CO) controlled environmental chamber (Tescor, Warminster, PA) in ambient conditions of 25°C and 40% relative humidity. Participants kept an exercise record for 2 days before and a dietary record for 24 h before the initial trial and replicated exercise and diet for these periods before the remaining trials. Additionally, participants abstained from exercise 24 hours before each trial. Following an overnight 12 hour fast, participants arrived at the laboratory in the early morning to complete testing. Upon arrival to the laboratory nude body mass was measured (CW-11, Ohaus Corporation, Pine Brook, NJ). Participants performed a 90 minute interval cycling protocol as follows: 1) 10-minute warm up at ~55% VO_{2max}, 2) series of ten intervals, which included two minutes at approximately 80% VO_{2max} followed by four minutes at ~50% VO_{2max}, 3) after the series of 10 intervals, completing 12 minutes at ~60% VO_{2max} followed by 10 minutes at ~50% VO_{2max}. Immediately after the ride, subjects toweled off and changed into dry clothes. Participants remained in a sitting position throughout the 6 hour recovery period. Participants received a liquid carbohydrate beverage immediately prior to entering the chamber (1.2 g•kg^{-1}) and solid feedings (1.28 g•kg^{-1} carbohydrate, 0.15 g•kg^{-1} fat, and 0.29 g•kg^{-1} protein) at 2 and 4 hours into recovery. Nude body mass was measured following the ride and at the end of the 6 hour

recovery period. Participants consumed 8 ml•kg^{-1} of water during the ride and ad-libitum water intake during the recovery.

Biopsies. Muscle biopsies were taken before and after exercise, and at the end of the 6 hour recovery period for each trial. Biopsies were taken from the *vastus lateralis* muscle using a 5 mm Bergstrom percutaneous muscle biopsy needle with the aid of suction (2). All subsequent biopsies during a given trial were obtained from the same leg using a separate incision 2 cm proximal to the previous biopsy. After excess blood, connective tissue, and fat were removed, tissue samples were stored in RNA Later or immersed in liquid nitrogen and stored at -80°C for later analysis.

Blood Samples. A 10 mL blood sample was taken from an antecubital vein before and after exercise and at 2, 4, and 6 hours during recovery. Immediately, 2 capillary tubes were filled with 100ul of blood each and placed in a hematocrit centrifuge for 3 minutes, 15,290 x g (A13, Jouan, Winchester, VA) for analysis of hematocrit. The proportion of blood cells to serum was measured in each tube and the average of the two measures was used to determine hematocrit. The remainder of the blood sample was then centrifuged at 7500 x g for 20 minutes at 4°C (MR 22i, Jouan, Winchester, VA). Plasma was removed and stored at -80˚C until subsequent analyses.

Respiratory Parameters. Expired gases were measured during recovery at 2:30, 4:30, and 5:55 to evaluate differences in oxygen consumption and respiratory function among trials. Each collection was collected for 5 min, with the last 3 min averaged to represent the sample period.

Pulse Oximetry. Blood oxygen saturation was evaluated before and after exercise, and during recovery at 0:30, 2:30, 4:30, and 6:00 using a pulse oximeter (Nonin Onyx II 9550, Plymouth, MN).

Analysis

Skeletal Muscle RNA isolation. An 8-20 mg piece of skeletal muscle will be homogenized in 800ul of Trizol using an electric homogenizer. The samples are then incubated at room temperature for 5 minutes after which 200ul of chloroform per 1000ul of Trizol is added and shaken vigorously by hand. After an additional incubation at room temperature for 2-3 minutes the samples are centrifuged at 12,000 g for 15 minutes and the aqueous phase was transferred to a fresh tube. The RNA is then precipitated by adding 500ul of isopropyl alcohol pre 1000ul of initial Trizol and incubated overnight at -20°C. The next morning samples are centrifuged at 12,000g for 10 minutes at 4°C and the RNA is washed by removing the supernatant and adding 1000ul of 75% ethanol per 1000ul of initial Trizol. The samples are then mixed by vortex and centrifuged at 7,500g for 5 minutes at 4°C. The RNA is then redissolved in 100 ul RNase-free water after the supernatant is removed and the RNA pellet was dried. The RNA is then cleaned using the RNeasy mini kit (Qiagen) according to the manufactures protocol using the additional DNase digestion step (DNA mini kit, Qiagen). RNA is then quantified using a nano-spectrophotometer.

cDNA synthesis. First-strand cDNA synthesis is achieved using Superscript-fist strand kit (Invitrogen) according to the manufactures protocol. Each sample within a given subject will contain the same amount of RNA (400 – 1000 ng). The resulting cDNA was then diluted using RNase free water in order to have adequate volume for RT-PCR and frozen for later RT-PCR analysis.

Real time RT-PCR. Each 25ul reaction volume will contain 500nM primers, 250nM probe (PrimeTime qPCR assay, Integrated DNA technologies), 1x FastStart TaqMan Probe master (Roche Applied Science), and 2.5 ul of sample cDNA. PCR will then run using the Bio-Rad I Cycler iQ5 Real-Time PCR Detection system (Bio-Rad) using a 2-step Roche protocol.

Oxidative Stress. Plasma aliquots were assayed for an oxidative stress biomarker panel used previously by our group (6,8). Two biomarkers, plasma trolox-equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant potential (FRAP), were chosen to evaluate blood plasma antioxidant capacity. Oxidative damage was evaluated in plasma by determination of protein carbonyls and lipid hydroperoxides content.

Antioxidant Capacity. Plasma antioxidant capacity was measured by the plasma trolox-equivalent antioxidant capacity technique whereby a radical cation of the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) reaction is quenched by sample-specific antioxidant fortifications (5). Total plasma antioxidant potential was determined by the ferric reducing antioxidant potential assay according to the methodology of (1). Both TEAC and FRAP assays result in quantifiable colorimetric solutions which are visualized spectrophotometrically. Lipid peroxidation was determined by the ferrous oxidation-xylenol orange assay (7). In brief, ferrous ions are oxidized by lipid hydroperoxides to ferric ions and subsequently react with the ferrous sensitive dye containing xylenol orange. In the presence of lipid hydroperoxides, this reaction forms a spectrophotometrically quantifiable complex. Protein carbonyls were analyzed using a commercially available ELISA kit (Zentech Technology, Dunedin, New Zealand). All assays were performed in triplicate and exhibited within sample coefficients of variation between 2% and 5%. Prior to analysis, all plasma samples were assayed in quadruplicate for protein concentration based on the methods of Bradford et al, (1976) and adjusted to 4 mg/ml protein using a phosphate buffer (4). All oxidative stress biomarkers were normalized for plasma volume shifts experienced during the three trials.

Statistics

Muscle glycogen, substrate utilization, mRNA of metabolic genes and oxidative stress markers were analyzed using a repeated measure ANOVA (trial*time). A probability of type I error less than 5% was considered significant ($p<0.05$). All data is reported as means ± SE.

References (methodology):

1. Benzie, IF, Strain, JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* 239: 70-76, 1996.

2. Bergstrom, J. Muscle electrolytes in man. *Scand J Clin Lab Invest* 14: 7-110, 1962.

3. Boren, H, Kory, RC, Syner, JC. The Veteran's Administration-Army cooperative study of pulmonary function: II. The lung volume and its subdivisions in normal men. *Am J Med* 41: 96-114, 1966.

4. Bradford, MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.

5. Cao, G, Russell, RM, Lischner, N, Prior, RL. Serum antioxidant capacity is increased by consumption of strawberries, spinach, red wine or vitamin C in elderly women. *J Nutr* 128: 2383-2390, 1998.

6. Hudson, MB, Hosick, PA, McCaulley, GO, Schrieber, L, Wrieden, J, McAnulty, SR, Triplett, NT, McBride, JM, Quindry, JC. The effect of resistance exercise on humoral markers of oxidative stress. *Med Sci Sports Exerc* 40: 542-548, 2008.

7. Nourooz-Zadeh, J, Tajaddini-Sarmadi, J, Wolff, SP. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. *Anal Biochem* 220: 403-409., 1994.

8. Quindry, JC, Stone, WL, King, J, Broeder, CE. The effects of acute exercise on neutrophils and plasma oxidative stress. *Med Sci Sports Exerc* 35: 1139-1145, 2003.

9. Siri, WE. Body composition from fluid spaces and density: analysis of methods. 1961. *Nutrition* 9: 480-491; discussion 480, 492, 1993.

RESULTS:

Gene Expression:
Gene expression for mitochondrial biogenesis related genes was reduced with hypoxia (simulated, 5000 m elevation) in 8 of 12 genes ($p < 0.05$, figures 1-4) as compared to normoxia (975 m elevation). The other 4 genes were reduced, but not to a significant level ($p > 0.05$).

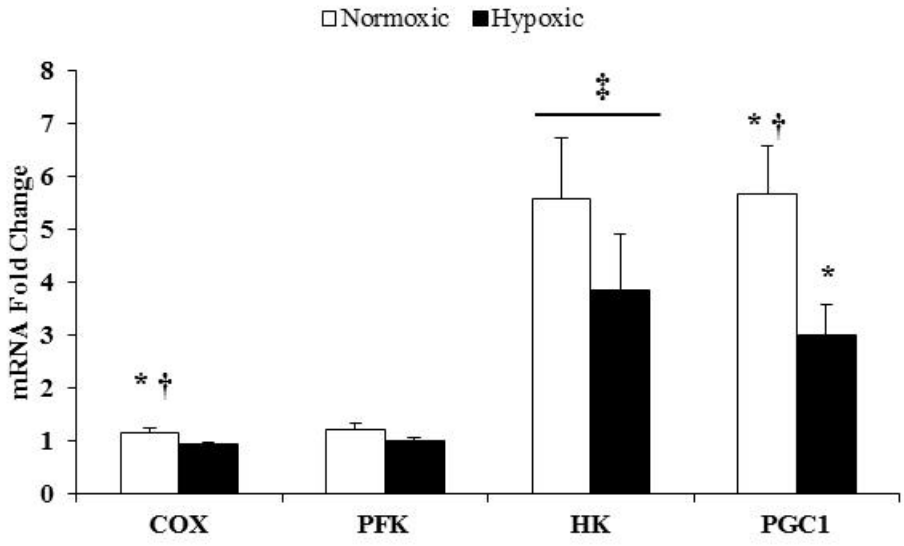

Figure 1. Metabolic gene expression six hours after exercise for normoxic and hypoxic conditions. Values are expressed as means ± SEM. * $p < 0.05$ from pre; † $p < 0.05$ from the hypoxic trial, ‡ $p < 0.05$ from pre (main effect of trial). COX, cytochrome c oxidase subunit 4; PFK, phoshofructokinase; HK, hexokinase; PGC1, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

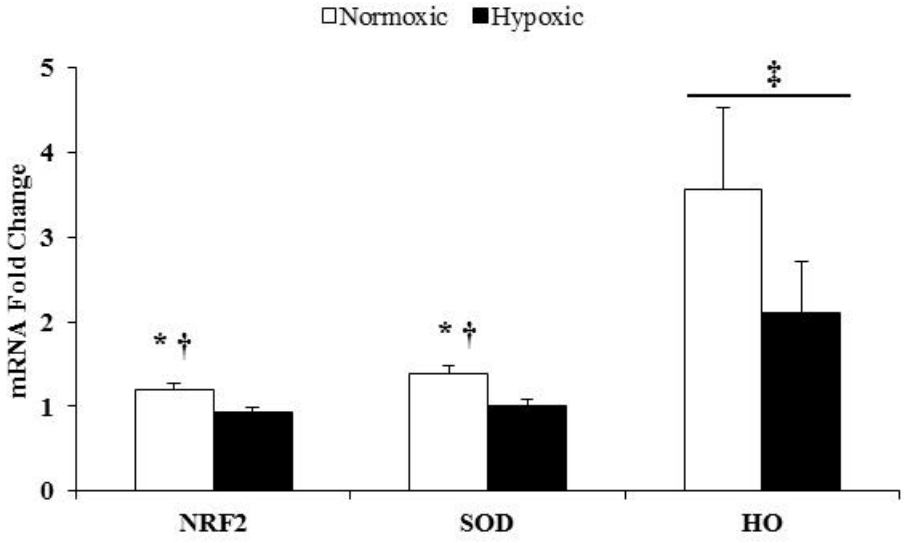

Figure 2. Oxidative stress related gene expression six hours after exercise for normoxic and hypoxic conditions. Values are expressed as means ± SEM. * $p < 0.05$ from pre; † $p < 0.05$

from the hypoxic trial, ‡ $p < 0.05$ from pre (main effect of trial). NRF2, nuclear factor (erythroid-derived 2)-like 2 also known as NFE2L2; SOD, superoxide dismutase 2; HO, heme oxygenase 1.

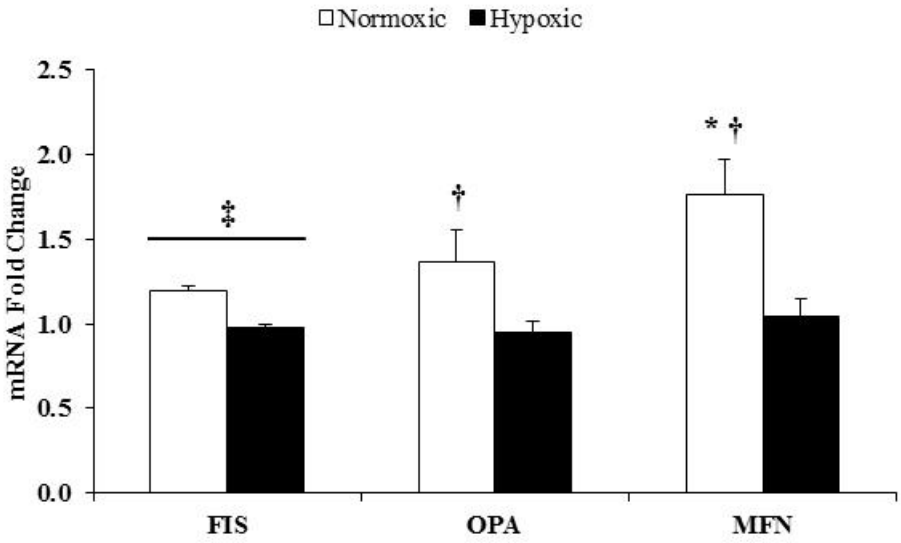

Figure 3. Mitochondrial morphology related gene expression six hours after exercise for normoxic and hypoxic conditions. Values are expressed as means ± SEM. * $p < 0.05$ from pre; † $p < 0.05$ from the hypoxic trial, ‡ $p < 0.05$ from pre (main effect of trial). FIS, mitochondrial fission protein 1; OPA, optic atrophy 1; MFN, mitofusin 2.

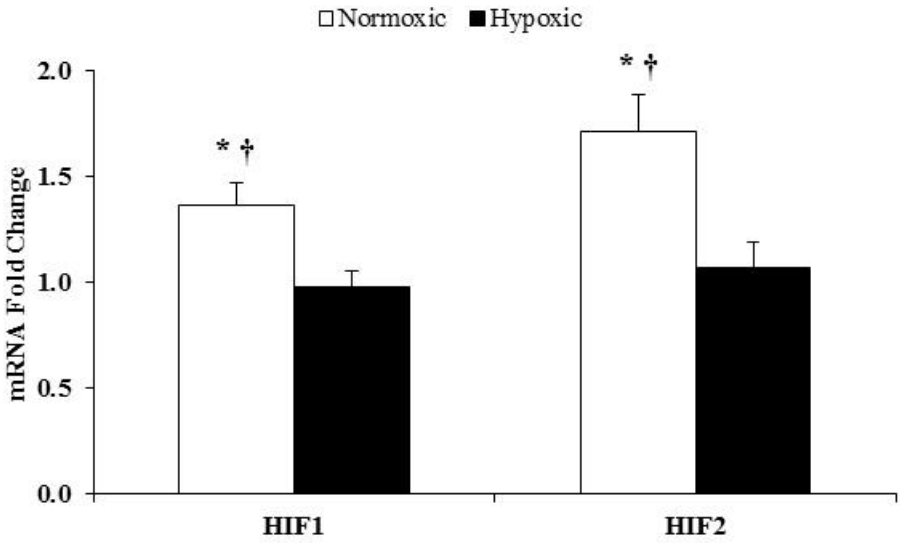

Figure 4. Hypoxia regulation gene expression six hours after exercise for normoxic and hypoxic conditions. Values are expressed as means ± SEM. * $p < 0.05$ from pre; † $p < 0.05$ from the hypoxic trial. HIF1, hypoxia inducible factor 1 alpha subunit; HIF2, hypoxia inducible factor 2 alpha subunit.

Oxidative stress:

Trolox equivalent antioxidant capacity of plasma (TEAC): Time (p=0.044, Pre vs Post, 1Hr; 6Hr vs 1Hr, 2Hr) and trial (p=0.021) main effects were present. Significant trial differences were present between Normoxic and Hypoxic recovery at 1Hr, 2Hr, and 6Hr recovery periods. Based on the fact that plasma TEAC is heavily influenced by plasma urate concentrations, this finding likely reflects increased purine metabolism during exercise. The metabolic pathway responsible for urate production, reflected by increased TEAC values, results in the production of reactive oxygen species during two enzymatically catalyzed reactions. Given the similarities to previous findings with similar study designs, we interpret this finding to indicate that exercise elicited an increase in oxidative stress during recovery, while hypoxic recovery attenuated this response.

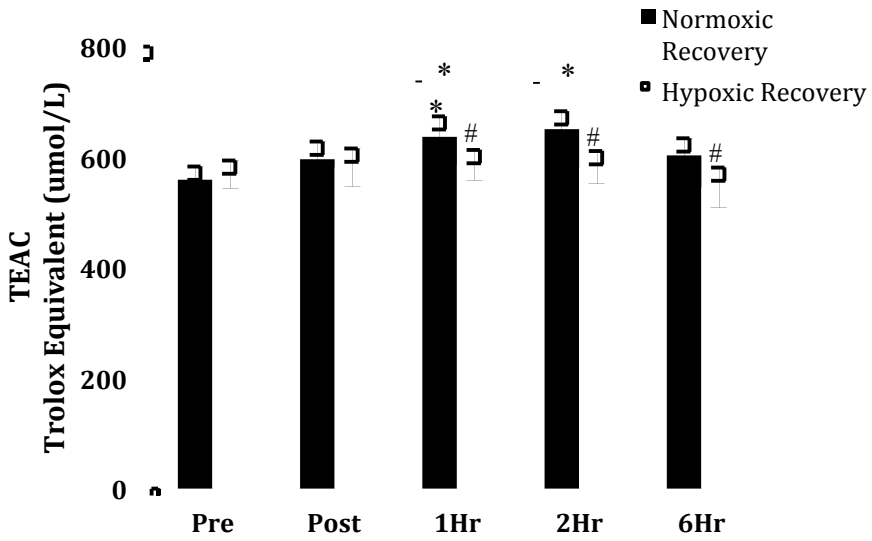

Figure 5. TEAC values pre, post, and under hypoxic recovery stress. Data are means +/- SEM; * denotes significantly different from Pre, # denotes significantly different from the Normoxic Recovery condition.

Ferric reducing ability of plasma (FRAP): Time (p=0.031, Post vs 6Hr) main effects and interaction effects (p=0.030) were present. Pre-Post differences approached significance (p=0.069). Significant trial differences were present between Normoxic and Hypoxic recovery at 6Hr recovery period with differences approaching significance 1Hr following exercise (p=0.072). Plasma FRAP values at the 6Hr time point were significantly lower than Pre during Hypoxic Recovery (p=0.020). These findings, while less conclusive than TEAC, generally support TEAC results suggesting that redox responses to Hypoxic Recovery were altered as compared to the Normoxic Recovery trial. In support, analysis of the percent change in plasma FRAP (Time main effect p=0.032; Trial main effect p=0.066) was observed including a significant drop in the percent change in at the 6Hr recovery time point (p=0.045) from Hypoxic Recovery samples.

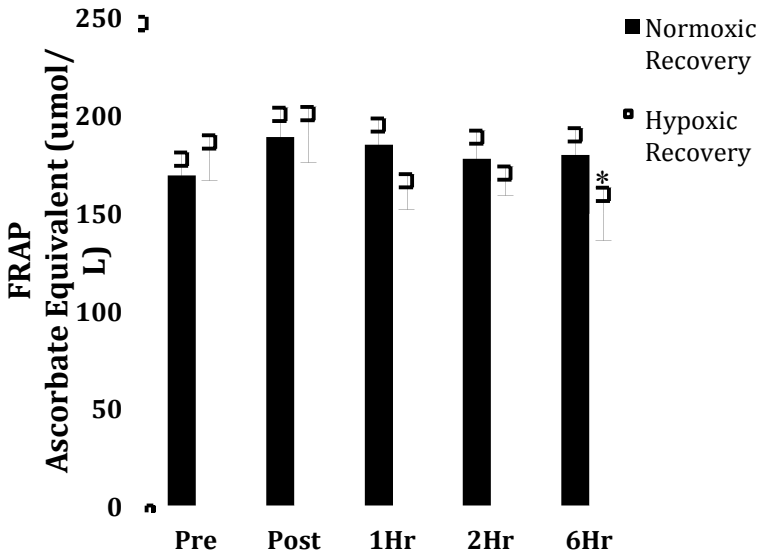

Figure 6. FRAP values pre, post, and under hypoxic recovery stress. Data are means +/- SEM; * denotes significantly different from Pre.

Lipid hydroperoxides (LOOH): Time main effects (p=0.041, Post vs all other time points) were observed for plasma LOOH. Sample variances were unusually large for this investigation and cannot be explained currently. While numeric differences between means suggest elevations in plasma LOOH following Normoxic Recovery vs Hypoxic Recovery, this conclusion was not supported statistically.

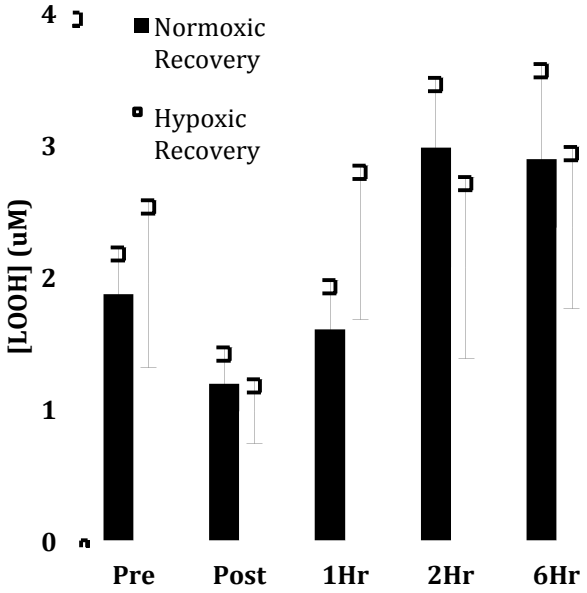

Figure 7. LOOH concentrations pre, post, and under hypoxic recovery stress. Data are means +/- SEM.

Protein carbonyls (PC): Time main effects (p=0.044, Post vs all other recovery time points) were observed for plasma PC. The percent change in plasma PC support findings with

absolute values in that 1Hr (p=0.005) were elevated as compared to Post (Post-2Hr, p=0.069). The percent increase over Pre values was most notable at 1Hr into the Normoxic Recovery (p=0.004) where an average increase of 16% was observed. As with plasma LOOH, examination of significant differences between trials is not warranted statistically, however, examination of post hoc analyses reveals an increase in 1Hr post Hypoxic Recovery as compared to the immediately post value.

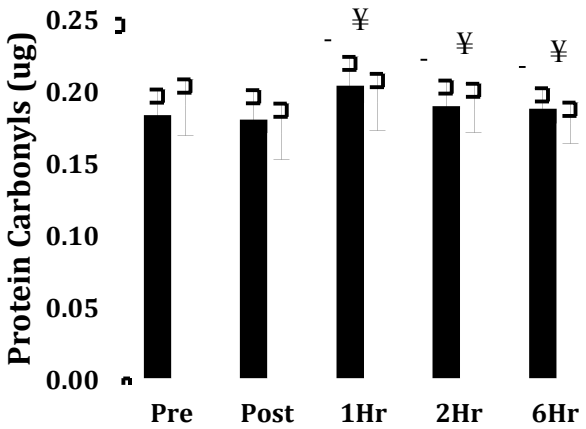

Figure 8. Protein Carbonyls pre, post, and under hypoxic recovery stress. Data are means +/- SEM; ¥ denotes significantly different from Post.

Interleukin-6 (IL-6): No main effects (time, p=0.484; trial, p=0.327) were not present for plasma IL-6 changes. Note that values required a polynomial regression. Notably, however, these IL-6 values were plagued by several subjects for which a consistent order of magnitude difference was noted for all trials and time points. As such we performed the percentage increase analyses here too and observed a significant trial difference (p=0.013) in that the post exercise increase in plasma IL-6 was significantly higher during the Hypoxic Recovery as compared to the Normoxic Recovery.

Summary:

Gene Expression:
The novel outcome of this investigation was the reduced gene expression of mitochondrial related genes with a very robust hypoxic intervention. Initially, this would appear to be contradictory to previous research that has shown increased mitochondrial biogenesis with altitude/hypoxic exposure (4, 6, 10, 14, 29, 30, 39, 40). However, these investigations did not investigate the effects of an aggressive hypoxic stimulus. At higher altitudes mitochondrial related function has been shown to decline (7, 9, 18, 19, 22, 27, 28).

From this, it may be speculated that a mild hypoxic stress may produce beneficial mitochondrial adaptations. In contrast, exposure to more extreme hypoxia may diminish the degree of mitochondrial adaptation. This speculation may explain the ambiguity of previous research on metabolic adaptations with altitude.

Future research should investigate a dose response relationship between the degree of hypoxia and the gene expression of mitochondrial associated markers. We hypothesize that there may be a critical altitude at which enhanced mitochondrial adaptation can occur after which diminished response to the magnitude of inhibition of response occurs.

Oxidative Stress:
The collective effect of these data strongly suggest that the exercise stimulus elicitied an oxidative stress response that became evident during the recovery period. The interesting discovery is that Hypoxic Recovery appeard to blunt many of these findings as compared to the Normoxic Recovery.

Examination of the data do raise questions about why some of the typical post exercise findings were not observed in the current samples. However, some of the expected common post exercise increases in these markers were not statistically significant dispite trends. Some of these findings may related to to hydration status of the subjects during the recovery period.

The decreased oxidative stress that we observed may provide the mechanism by which mitochondrial stimuli is reduced. Oxidative stress may be stimuli for mitochondrial adaptation that does not continue to increase in a linear manner at very high levels of hypoxia.

KEY RESEARCH ACCOMPLISHMENTS:

- Statement of work tasks 5-6 have been completed.
- Task 7a has been completed.
- The phase 3 laboratory study has been revised based on initial findings from year 1 and 2 efforts and has received University IRB approval. HRPO submission will occur by November 2, 2012.
- A manuscript focusing on the oxidative stress findings from study 2 is being prepared.

- A manuscript focusing on the mitochondrial gene response findings from study 2 is being prepared.
- Additional abstracts are being prepared for submission and presentation at the National ACSM meeting, June 2013.

REPORTABLE OUTCOMES:

- A manuscript focusing on the oxidative stress findings from study 2 is being prepared.
- A manuscript focusing on the mitochondrial gene response findings from study 2 is being prepared.
- Additional abstracts are being prepared for submission and presentation at the National ACSM meeting, June 2013.

CONCLUSIONS:

The purpose of this research determines how hypoxia interacts with exercise and recovery to yield various metabolic responses that may affect performance, performance at high-altitude, and the initial phase of metabolic training adaptations critical to mission success. In the present study design, participants completed two 90-minute interval exercise sessions (cycle ergometer) under normal lab (normoxic) conditions (975 m). After the exercise periods, subjects recovered in a climate, controlled chamber (Tescor) for 6 hours at either normoxic (975 m) or hypoxic (5000 m) conditions. During year 1, recovery occurred at 3000 m and there were minimal differences in the mitochondria gene expression between 975 and 3000 m. Therefore, for this phase of our investigation series, we increased the degree of hypoxia.

The two trials were completed using a randomized, counterbalanced, cross over design over the span of 3 weeks, with a minimum of 7 days between trials. Muscle biopsies were obtained prior to and immediately following the 90-minute exercise period and again after 6 hours of recovery. Samples were analyzed for a select series of mitochondrial genes. Additional blood samples obtained at similar intervals were analyzed to account for changes in plasma volume and select markers of oxidative stress.

The most notable finding from the present investigation was the reduced gene expression of mitochondrial related genes as a result of the hypoxic intervention. As indicated above, this appears to be in contrast with prior research that has demonstrated an increase in markers of mitochondrial biogenesis with mild altitude/hypoxic exposure (4, 6, 10, 14, 29, 30, 39, 40). However, our data is in agreement and expands other research by demonstrating an overall decline in mitochondrial function at extreme or more aggressive altitudes (7, 9, 18, 19, 22, 27, 28). When these data are coupled with data from year one, it suggests that a mild hypoxic stress may produce beneficial mitochondrial adaptations regardless of the exercise stress. In contrast, exposure to more extreme hypoxia may diminish the degree of mitochondrial adaptation.

These data further suggest that future work (year 3) should investigate a dose response relationship between the degree of hypoxia and the gene expression of mitochondrial associated markers. We hypothesize that there may be a specific degree of hypoxia after which the degree of mitochondrial adaptation is diminished. For year 3, we have modified our existing chamber to accommodate evaluate in a hyperoxic environment (allowing us to mimic the physiological conditions of sea level).

From a practical standpoint, the data from year 1 demonstrated that acute exercise stress under normoxic or hypoxic conditions results in similar physiological responses that may set the stage for subsequent adaptations in the mitochondria. In contrast, these new data from year 2 suggest that under extreme hypoxic conditions (simulating 5000 m), the potential for mitochondrial biogenesis is diminished. Year 1 results suggested that the responses of metabolic and mitochondrial genes and markers of oxidative stress are driven by exercise intensity and that potential for metabolic adaptations are similar across environments. However, these results are not inclusive of higher altitudes based on these year 2 findings.

In concert, these results suggest that to maximize the metabolic responses associated with arduous exercise stress, participants should not be exposed to hypoxic conditions or altitudes that exceed 3000 m.

For example, if individuals are attempting to gain both metabolic adaptations from aggressive training strategies, the use of hypoxic tent systems or chambers (commonly used to enhance acclimation to altitude for theoretical mission preparedness) should be used with some reservation.

Individuals, trainers and medical personnel should recognize that the metabolic adaptations associated with exercise training may be compromised if individuals are exposed to high altitudes (5000 m). At this point, it is unclear if altitudes between 3000 and 5000 m will alter the initial metabolic response to exercise as measured using markers of mitochondrial biogenesis. It is apparent from these data that the goals of enhancing acclimation to hypoxic environments are counter to maximizing the activation of mitochondrial development.

References (introduction, summary, conclusions):

1. Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, Kelly DP and Holloszy JO. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. *FASEB J.* 16: 14: 1879-1886, 2002.

2. Bach D, Naon D, Pich S, Soriano FX, Vega N, Rieusset J, Laville M, Guillet C, Boirie Y, Wallberg-Henriksson H, Manco M, Calvani M, Castagneto M, Palacin M, Mingrone G, Zierath JR, Vidal H and Zorzano A. Expression of Mfn2, the Charcot-Marie-Tooth neuropathy type 2A gene, in human skeletal muscle: effects of type 2 diabetes, obesity, weight loss, and the regulatory role of tumor necrosis factor alpha and interleukin-6. *Diabetes* 54: 9: 2685-2693, 2005.

3. Bach D, Pich S, Soriano FX, Vega N, Baumgartner B, Oriola J, Daugaard JR, Lloberas J, Camps M, Zierath JR, Rabasa-Lhoret R, Wallberg-Henriksson H, Laville M, Palacin M, Vidal H, Rivera F, Brand M and Zorzano A. Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. A novel regulatory mechanism altered in obesity. *J.Biol.Chem.* 278: 19: 17190-17197, 2003.

4. Bailey DM, Davies B and Baker J. Training in hypoxia: modulation of metabolic and cardiovascular risk factors in men. *Med.Sci.Sports Exerc.* 32: 6: 1058-1066, 2000.

5. Brooks GA, Butterfield GE, Wolfe RR, Groves BM, Mazzeo RS, Sutton JR, Wolfel EE and Reeves JT. Increased dependence on blood glucose after acclimatization to 4,300 m. *J.Appl.Physiol.* 70: 2: 919-927, 1991.

6. Desplanches D, Hoppeler H, Linossier MT, Denis C, Claassen H, Dormois D, Lacour JR and Geyssant A. Effects of training in normoxia and normobaric hypoxia on human muscle ultrastructure. *Pflugers Arch.* 425: 3-4: 263-267, 1993.

7. Favier R, Spielvogel H, Desplanches D, Ferretti G, Kayser B and Hoppeler H. Maximal exercise performance in chronic hypoxia and acute normoxia in high-altitude natives. *J.Appl.Physiol.* 78: 5: 1868-1874, 1995.

8. Feldser D, Agani F, Iyer NV, Pak B, Ferreira G and Semenza GL. Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. *Cancer Res.* 59: 16: 3915-3918, 1999.

9. Ferretti G, Boutellier U, Pendergast DR, Moia C, Minetti AE, Howald H and di Prampero PE. Oxygen transport system before and after exposure to chronic hypoxia. *Int.J.Sports Med.* 11 Suppl 1: S15-20, 1990.

10. Gastaldi G, Russell A, Golay A, Giacobino JP, Habicht F, Barthassat V, Muzzin P and Bobbioni-Harsch E. Upregulation of peroxisome proliferator-activated receptor gamma coactivator gene (PGC1A) during weight loss is related to insulin sensitivity but not to energy expenditure. *Diabetologia* 50: 11: 2348-2355, 2007.

11. Geiser J, Vogt M, Billeter R, Zuleger C, Belforti F and Hoppeler H. Training high--living low: changes of aerobic performance and muscle structure with training at simulated altitude. *Int.J.Sports Med.* 22: 8: 579-585, 2001.

12. Gorlach A, Diebold I, Schini-Kerth VB, Berchner-Pfannschmidt U, Roth U, Brandes RP, Kietzmann T and Busse R. Thrombin activates the hypoxia-inducible factor-1 signaling pathway in vascular smooth muscle cells: Role of the p22(phox)-containing NADPH oxidase. *Circ.Res.* 89: 1: 47-54, 2001.

13. Gracey AY, Troll JV and Somero GN. Hypoxia-induced gene expression profiling in the euryoxic fish Gillichthys mirabilis. *Proc.Natl.Acad.Sci.U.S.A.* 98: 4: 1993-1998, 2001.

14. Green H, MacDougall J, Tarnopolsky M and Melissa NL. Downregulation of Na+-K+-ATPase pumps in skeletal muscle with training in normobaric hypoxia. *J.Appl.Physiol.* 86: 5: 1745-1748, 1999.

15. Hellwig-Burgel T, Rutkowski K, Metzen E, Fandrey J and Jelkmann W. Interleukin-1beta and tumor necrosis factor-alpha stimulate DNA binding of hypoxia-inducible factor-1. *Blood* 94: 5: 1561-1567, 1999.

16. Holloszy JO. Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J.Biol.Chem.* 242: 9: 2278-2282, 1967.

17. Hoppeler H and Fluck M. Plasticity of skeletal muscle mitochondria: structure and function. *Med.Sci.Sports Exerc.* 35: 1: 95-104, 2003.

18. Hoppeler H, Kleinert E, Schlegel C, Claassen H, Howald H, Kayar SR and Cerretelli P. Morphological adaptations of human skeletal muscle to chronic hypoxia. *Int.J.Sports Med.* 11 Suppl 1: S3-9, 1990.

19. Howald H, Pette D, Simoneau JA, Uber A, Hoppeler H and Cerretelli P. Effect of chronic hypoxia on muscle enzyme activities. *Int.J.Sports Med.* 11 Suppl 1: S10-4, 1990.

20. Jewell UR, Kvietikova I, Scheid A, Bauer C, Wenger RH and Gassmann M. Induction of HIF-1alpha in response to hypoxia is instantaneous. *FASEB J.* 15: 7: 1312-1314, 2001.

21. Jezek P and Plecita-Hlavata L. Mitochondrial reticulum network dynamics in relation to oxidative stress, redox regulation, and hypoxia. *Int.J.Biochem.Cell Biol.* 41: 10: 1790-1804, 2009.

22. Kayser B, Hoppeler H, Claassen H and Cerretelli P. Muscle structure and performance capacity of Himalayan Sherpas. *J.Appl.Physiol.* 70: 5: 1938-1942, 1991.

23. Koves TR, Li P, An J, Akimoto T, Slentz D, Ilkayeva O, Dohm GL, Yan Z, Newgard CB and Muoio DM. Peroxisome proliferator-activated receptor-gamma co-activator 1alpha-

mediated metabolic remodeling of skeletal myocytes mimics exercise training and reverses lipid-induced mitochondrial inefficiency. *J.Biol.Chem.* 280: 39: 33588-33598, 2005.

24. Laughner E, Taghavi P, Chiles K, Mahon PC and Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol.Cell.Biol.* 21: 12: 3995-4004, 2001.

25. Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R and Spiegelman BM. Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* 418: 6899: 797-801, 2002.

26. Lundby C, Gassmann M and Pilegaard H. Regular endurance training reduces the exercise induced HIF-1alpha and HIF-2alpha mRNA expression in human skeletal muscle in normoxic conditions. *Eur.J.Appl.Physiol.* 96: 4: 363-369, 2006.

27. MacDougall JD, Green HJ, Sutton JR, Coates G, Cymerman A, Young P and Houston CS. Operation Everest II: structural adaptations in skeletal muscle in response to extreme simulated altitude. *Acta Physiol.Scand.* 142: 3: 421-427, 1991.

28. Martinelli M, Winterhalder R, Cerretelli P, Howald H and Hoppeler H. Muscle lipofuscin content and satellite cell volume is increased after high altitude exposure in humans. *Experientia* 46: 7: 672-676, 1990.

29. Meeuwsen T, Hendriksen IJ and Holewijn M. Training-induced increases in sea-level performance are enhanced by acute intermittent hypobaric hypoxia. *Eur.J.Appl.Physiol.* 84: 4: 283-290, 2001.

30. Melissa L, MacDougall JD, Tarnopolsky MA, Cipriano N and Green HJ. Skeletal muscle adaptations to training under normobaric hypoxic versus normoxic conditions. *Med.Sci.Sports Exerc.* 29: 2: 238-243, 1997.

31. Mingrone G, Manco M, Calvani M, Castagneto M, Naon D and Zorzano A. Could the low level of expression of the gene encoding skeletal muscle mitofusin-2 account for the metabolic inflexibility of obesity? *Diabetologia* 48: 10: 2108-2114, 2005.

32. Norrbom J, Sundberg CJ, Ameln H, Kraus WE, Jansson E and Gustafsson T. PGC-1alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. *J.Appl.Physiol.* 96: 1: 189-194, 2004.

33. Pages G, Milanini J, Richard DE, Berra E, Gothie E, Vinals F and Pouyssegur J. Signaling angiogenesis via p42/p44 MAP kinase cascade. *Ann.N.Y.Acad.Sci.* 902: 187-200, 2000.

34. REYNAFARJE B. Myoglobin content and enzymatic activity of human skeletal muscle--their relation with the process of adaptation to high altitude. *Tech.Doc.Rep.SAMTDR USAF Sch.Aerosp.Med.* SAM-TDR-62-89: 8p, 1962.

35. Richard DE, Berra E and Pouyssegur J. Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. *J.Biol.Chem.* 275: 35: 26765-26771, 2000.

36. Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. *Annu.Rev.Cell Dev.Biol.* 15: 551-578, 1999.

37. Slivka DR, Dumke CL, Hailes WS, Cuddy JS and Ruby BC. Substrate use and biochemical response to a 3,211-km bicycle tour in trained cyclists. *Eur.J.Appl.Physiol.* 112: 5: 1621-1630, 2012.

38. Stroka DM, Burkhardt T, Desbaillets I, Wenger RH, Neil DA, Bauer C, Gassmann M and Candinas D. HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. *FASEB J.* 15: 13: 2445-2453, 2001.

39. Terrados N, Melichna J, Sylven C, Jansson E and Kaijser L. Effects of training at simulated altitude on performance and muscle metabolic capacity in competitive road cyclists. *Eur.J.Appl.Physiol.Occup.Physiol.* 57: 2: 203-209, 1988.

40. Vogt M, Puntschart A, Geiser J, Zuleger C, Billeter R and Hoppeler H. Molecular adaptations in human skeletal muscle to endurance training under simulated hypoxic conditions. *J.Appl.Physiol.* 91: 1: 173-182, 2001.

41. Wende AR, Schaeffer PJ, Parker GJ, Zechner C, Han DH, Chen MM, Hancock CR, Lehman JJ, Huss JM, McClain DA, Holloszy JO and Kelly DP. A role for the transcriptional coactivator PGC-1alpha in muscle refueling. *J.Biol.Chem.* 282: 50: 36642-36651, 2007.

42. Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW and Semenza GL. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res.* 60: 6: 1541-1545, 2000.

43. Zoll J, Ponsot E, Dufour S, Doutreleau S, Ventura-Clapier R, Vogt M, Hoppeler H, Richard R and Fluck M. Exercise training in normobaric hypoxia in endurance runners. III. Muscular adjustments of selected gene transcripts. *J.Appl.Physiol.* 100: 4: 1258-1266, 2006.

APPENDICES:

Appendix 1: UM IRB Approval

Form RA-108
(Rev. 7/11)

THE UNIVERSITY OF MONTANA-MISSOULA
Institutional Review Board (IRB)
for the Use of Human Subjects in Research
CHECKLIST / APPLICATION

IRB Protocol No.: 156-11

At The University of Montana (UM), the Institutional Review Board (IRB) is the institutional review body responsible for oversight of all research activities involving human subjects outlined in the U.S. Department of Health and Human Services Office of Human Research Protection (www.hhs.gov/ohrp) and the National Institutes of Health, Inclusion of Children Policy Implementation (http://grants.nih.gov/grants/funding/children/children.htm).

Instructions: A separate registration form must be submitted for each project. IRB proposals are approved for three years and must be continued annually. Faculty and students may email the completed form as a Word document to *IRB@umontana.edu.* or submit a hardcopy to the Office of the Vice President for Research & Development, University Hall 116. Student applications must be accompanied by email authorization by the supervising faculty member or a signed hard copy.

All fields must be completed. If an item does not apply to this project, write in: n/a.

1. Administrative Information

Project Title: The impact of hypoxia and carbohydrate feedings on recovery from exercise	
Principal Investigator: Brent C. Ruby, PhD	Title: Professor
Email address: brent.ruby@mso.umt.edu	
Work Phone: 406-243-2117	Cell Phone: 406-396-4382
Department: HHP	Office location: McGill 244

2. Human Subjects Protection Training

(All researchers, including faculty supervisors for student projects, must have completed a self-study course on protection of human research subjects ***within the last three years*** *(http://www.umt.edu/research/complianceinfo/IRB/) and be able to supply the "Certificate(s) of Completion" upon request. Add rows to table if needed.*

NAME and DEPT.	PI	CO-PI	Faculty Supervisor	Research Assistant	DATE COMPLETED Human Subjects Protection Course
Brent C. Ruby	X	☐	☐	☐	3/2009
Charles Dumke	☐	X	☐	☐	10/2008
John Cuddy	☐	☐	☐	X	3/2009
Walter Hailes	☐	☐	☐	X	2/2009

3. Project Funding

(If federally funded, you must submit a copy of the abstract.)

Is grant application currently under review at a grant funding agency? ☐Yes *(If yes, cite sponsor on ICF if applicable)* X No		Has grant proposal received approval and funding? X Yes *(If yes, cite sponsor on ICF if applicable)* ☐No		
Agency	Grant No.	Start Date	End Date	PI
Defense Medical Research and Development Program	W81XWH-10-2--0120	10/1/2010	9/30/2013	Brent Ruby
Is this part of a thesis or dissertation? ☐Yes X No If yes, whose? _____		If **yes**, date you successfully presented your proposal to your committee: n/a		

For UM-IRB Use Only

IRB Determination:

_____ Approved Exempt from Review, Exemption # ____ *(see memo)*
_____ Approved by Expedited Review, Category #___ *(see* ***Note to PI****)*
_____ Full IRB Determination
  _____ Approved *(see* ***Note to PI****)*
  _____ Conditional Approval *(see memo)* - IRB Chair Signature/Date: _____
    _____ Conditions Met *(see* ***Note to PI****)*
  _____ Resubmit Proposal *(see memo)*
  _____ Disapproved *(see memo)*

Risk Level: _____

*** Note to PI:** Study is approved for one year. Use any attached IRB-approved forms (signed/dated) as "masters" when preparing copies. If continuing beyond the expiration date, a continuation report must be submitted. Notify the IRB if any significant changes or unanticipated events occur. Notify the IRB in writing when the study is terminated

Final Approval by IRB Chair: _____ Date: 8-29-2011 Expires: 8-28-2012

The University of Montana

Montana Center for Work Physiology and Exercise Metabolism

SUBJECT INFORMATION AND CONSENT FORM

PROJECT IN BRIEF: The impact of hypoxia and carbohydrate feedings on recovery from exercise

SPONSOR: Defense Medical Research and Development Program, a Department of Defense (DOD) Organization

RESEARCHERS: Dr. Brent Ruby, PhD (406) 243-2117
Dr. Charles Dumke, PhD
John Cuddy
Walter Hailes

The University of Montana
Montana Center for Work Physiology and Exercise Metabolism
32 Campus Drive
McGill Hall – HHP
Missoula, MT 59812
(406) 243 – 2117 (Dr. Brent Ruby, PhD)

Please read the following information carefully and feel free to ask questions. Only sign the final page when you are satisfied procedures and risks have been sufficiently explained to you.

REQUIREMENTS

This research study requires that you meet the following criteria:

- Participants must be males between the ages of 18 and 40.

PURPOSE OF THE STUDY

The study is designed to address the issue in current hypoxia research of recovery after exercise. This study will address how recovering in a hypoxic environment may affect muscle glycogen, specific mitochondrial genes related to exercise training, and oxidative stress.

TEST PROCEDURES

3 VISITS TO THE LABORATORY WILL BE REQUIRED (18 HOURS), AS SUMMARIZED BELOW

Project funded by the Defense Medical Research and Development Program, a Department of Defense (DOD) organization

PRE TESTING (Visit 1)

1. A pre-screening assessment which involves a health/exercise questionnaire (Par-Q) and question regarding prior acute mountain sickness.
    a. Prior to any testing, you will complete a physical activity readiness questionnaire (PAR-Q) to screen for known risk factors of coronary heart disease.
    b. Prior to any testing, you will be excluded from the study if you have previously had serious acute mountain sickness.
2. If you successfully complete the PAR-Q, you will then provide written informed consent following the reading of this document.
3. A measure of percent body fat obtained using underwater weighing
    a. This test session will require that you do not eat for a minimum of 3 hours prior to the testing. Prior to the test, body weight will be recorded in your bathing suit. You will then be asked to complete between 3 – 6 underwater weighing procedures. The underwater weight requires that you are submersed in our weighing tank (similar to a hot tub) and that you maximally exhale as much air as possible while underwater. The underwater weight will be recorded within 2-4 seconds and then you will be signaled to surface. This procedure will be repeated until three measurements have been obtained that are within 100 grams of each other. A nose clip will be provided upon request. This test will take approximately 20 minutes.
4. A maximal cycle ergometer test to measure aerobic fitness
    a. This test will consist of cycling on a laboratory treadmill to volitional fatigue. The workload of the cycle ergometer will increase every three minutes and will progress to fatigue. You will be encouraged to continue until volitional fatigue, the point at which you can no longer continue cycling. During this test you will wear a nose clip and headgear that will support a mouthpiece. This will allow us to measure the amount of oxygen that the body uses during this exercise so we can determine the appropriate exercise intensities for your experimental trial rides. Heart rate will be measured using an elastic chest strap that is worn on the skin under your shirt around your chest. This test will take approximately 30 minutes. You will be asked to fast for approximately 3 hours prior to this test.

EXPERIMENTAL TRIALS (Visits 2 and 3)

Trial 1) cycling for 90 minutes at varying intensities in laboratory at 975 meters (Missoula, MT elevation) followed by 6 hour recovery in environmental chamber at 975 meters altitude
Trial 2) cycling for 90 minutes at varying intensities in laboratory at 975 meters (Missoula, MT elevation) followed by 6 hour recovery in environmental chamber at a simulated 5000 meters altitude
**m = meters above sea level*

Experimental Protocol
Following a controlled diet (with NO alcohol consumption) and exercise plan the day before and after an overnight fast, you will arrive to the laboratory in the early morning following a 12 hour

Project funded by the Defense Medical Research and Development Program, a Department of Defense (DOD) organization

fast. A blood sample will be taken from an arm vein in your forearm and a muscle biopsy will be taken from the *vastus lateralis* (quad muscle) before commencement of exercise (Pre). Trials will be completed in a randomized order, but you will complete each of the two trials (7-14 days in between trials). The exercise protocol will be completed on a cycle ergometer in the laboratory. You will complete a 10-minute warm up at approximately 55% peak VO_2. Thereafter, you will complete a series of ten intervals, which includes two minutes at approximately 80% peak VO_2 followed by four minutes at approximately 50% peak VO_2. After the series of 10 intervals, you will complete 8 minutes at 60% peak VO_2 followed by 12 minutes at 50% peak VO_2. Total cycle time will be 90 minutes. You will be provided ~600 mL of water during the ride. Immediately upon cessation of exercise, a blood sample will be drawn and muscle biopsy will be taken from a separate incision ~2 cm above the Pre muscle biopsy (Post). You will then void if necessary, have body weight measured, and change clothing into a standardized shorts and t-shirt. You will then recover for 6 hours (sitting or lying) in the environmental chamber [22°C (72°F), 40% relative humidity] (in a random selection, one day will be hypoxic 5000 meters, and the other normoxic 975 meters). During this 6 hour recovery you will be supervised at all times by research staff. You will be provided a carbohydrate beverage at 0 hours into the recovery, and a solid food feeding at 2 and 4 hours into the recovery. Each feeding will be at a carbohydrate amount of 1.2 g/kg of body weight. During the recovery, blood samples will be taken at 2, 4, and 6 hours; metabolic gas collections will be taken 30 minutes into the recovery, and at 2, 4, and 6 hours into the recovery. Water will be provided ad-libitum during the 6 hour recovery. Following the 6 hour recovery, you will have a muscle biopsy taken from a separate incision ~2 cm above the Post muscle biopsy and an additional blood sample.

Biopsies

A total of 6 (3 per trial x 2 trials) muscle biopsies (3 from each leg) will be obtained from the front of your thigh muscle (vastus lateralis, approximately 6 inches up from the kneecap on the lateral side of your thigh). The muscle biopsy procedure requires that the site be sterilized. After the site is cleaned, a small amount of local anesthesia (lidocaine) will be injected just under the skin surface. Additional small amounts of lidocaine will be injected around a small 1-inch area around the site on the leg. After the area is treated with the lidocaine (approximately 5 mL, 1% lidocaine), a small incision (approximately 1/4 inch long) will be made through the skin and the outer covering (fascia) of your muscle to a depth of approximately 3/4-1.5 inches. The biopsy needle will then be inserted through the incision and the sample obtained. After the sample is obtained, the site will be cleaned and closed with steri-strips and/or a single stitch and bandaid and wrapped with a compression bandage. The biopsy samples will be obtained a) before the exercise session, b) after the exercise session, and c) following the 6 hours of recovery (biopsies for each trial will be on the same leg, above the initial or previous sample). This will be repeated for the second trial using the opposite leg. The muscle biopsies will be used to evaluate alterations in specific mitochondrial genes and muscle carbohydrate utilization kinetics in response to physical activity. Latex free bandages will be provided if subjects have a known allergy to latex. All of the muscle biopsies will be conducted by Dr. Brent Ruby or Dr. Charles Dumke.

Blood Samples

A total of 10 blood samples (5 per trial) will be collected using a venipuncture technique. The site will be cleaned with alcohol prior to the blood draw, and wiped clean afterwards. These

Project funded by the Defense Medical Research and Development Program, a Department of Defense (DOD) organization

samples will be collected to measure blood glucose and insulin. All of the blood samples will be obtained under the direction of Dr. Brent Ruby or Dr. Charles Dumke. Blood samples will be taken before exercise, and then at intervals 0, 2, 4, and 6 hours into the recovery. ~10 mL will be drawn each time for a total of ~50 mL per trial. Blood samples will be used to evaluate alterations in oxidative stress biomarkers.

Carbohydrate Beverage/Solid Feedings
Immediately following exercise, you will be provided a standardized high-carbohydrate drink. You will be asked to consume this drink as quickly as possible without upsetting your stomach. The amount of carbohydrate consumed at each feeding time point (Post ride, 2 and 4 hours in recovery) will be 1.2 g/kg.

Metabolic Gas Measurements
Expiratory gasses will be measured during the 6 hour recovery at 30 min, 2, 4, and 6 hours. This requires you to breathe through a mouthpiece while wearing a nose clip, the same setup that will be used during the maximal oxygen uptake test.

Dietary and Activity Recall
For 24-hours before your first exercise trial you will be asked to record the foods and quantity that you consume. You are not allowed to consume any alcohol during this time period. For the second trial, you will consume the same foods and quantity of those foods that you consumed for the first trial. 2 days before your first trial day you can exercise as you wish, but this must be repeated at the same time of day and the same exercise prior to the second trial. For the 24-hours before each trial you cannot participate in any physical exercise.

Body Weight
Nude body weight will be measured in private on a calibrated scale. Weights will be taken before, during recovery, and after each trial.

Urine
You will be asked to void your bladder before each trial. After the initial void, urine will be collected in a disposable plastic container and urine volume will be measured for the duration of each trial.

Pulse Oximetry
Spot check measurements throughout the exercise trial and 6 hour recovery will be taken using a pulse oximeter to check for blood oxygen saturation. The measurements will be taken pre exercise, after cycling for 45 minutes, post exercise, and every hour during the 6 hour recovery.

RISKS AND DISCOMFORTS

1. Mild discomfort may result during and after the exercise. These discomforts include shortness of breath, tired or sore legs, nausea and possibility of vomiting.
2. Muscle soreness after the tests may occur as a result of the exercise, but should not persist.
3. Certain changes in body function take place when any person exercises. Some of these changes are normal and others are abnormal. Abnormal changes may occur in blood

Project funded by the Defense Medical Research and Development Program, a Department of Defense (DOD) organization

pressures, heart rate, heart rhythm or extreme shortness of breath. Very rare instances of heart attack have occurred. Every effort will be made to minimize possible problems by the preliminary evaluation and constant surveillance during testing. The laboratory has standard emergency procedures should any potential problems arise.

4. Symptoms of dehydration such as headache and general fatigue may result during and after the exercise.
5. You will be informed of any new findings that may affect your decision to remain in the study.
6. The muscle biopsy and blood sampling techniques may cause some local and temporary discomfort. It is normal to have the sensation of a deep tissue bruise around the site of the muscle biopsy. This pain should be manageable and not above the pain associated from a "charlie horse" type bruise. Risks involved with muscle biopsies include: nerve damage, moderate stiffness, hematoma, minimal scarring, bleeding, fainting, and seizure.
7. There is a minor risk of infection associated with blood sampling and the muscle biopsy. Should you notice unusual redness, swelling or drainage at the biopsy incision site or at the sites of the blood sampling sites you should seek medical attention and then notify Brent Ruby, study director.
8. There are minimal risks associated with the use of lidocaine (the local anesthetic). Risks include: pain at the injection site, dizziness, confusion, shakiness, visual changes, nausea, and unusually slow heart rate. The risk of a reaction to the lidocaine is extremely low (approximately 1/1,000,000). However to minimize this risk, no more than 9 mL of a 1% lidocaine solution will be used per biopsy. You will be excluded from participation if you have a known history of allergic reactions to local anesthetics.
9. During any of the exercise tests should symptoms, such as chest discomfort, unusual shortness of breath or other abnormal findings develop, the exercise physiologist conducting the research will terminate the test. Guidelines by the American College of Sports Medicine will be followed to determine when a test should be stopped. These symptoms include moderate to severe angina (chest pain), increased dizziness, shortness of breath, fatigue and your desire to stop.
10. During recovery in the 5000 meter simulated laboratory trial, you may experience acute mountain sickness and may experience the following symptoms: headache, fatigue, dyspnea, hyperventilation, gastrointestinal distress, and decreased thirst.

PAYMENT FOR PARTICIPATION

Payment will be according to the following scale:
Preliminary tests: $50
Experimental trial #1: $250
Experimental trial #2: $250

Therefore, upon completion of the entire study, you will be paid a total of $550. If you decide to withdraw at any time, you will be compensated for the test sessions you have completed.

BENEFITS OF PARTICIPATION

Project funded by the Defense Medical Research and Development Program, a Department of Defense (DOD) organization

1. The information from these tests will provide you with an accurate assessment of your aerobic fitness and body composition that can be compared with norms for your age and sport but may be of little benefit to your understanding of your personal fitness. There are no other direct benefits to the participants in the study.
2. There is no promise that you will receive any benefit as a result of taking part in this study.
3. The scientific benefit includes elucidating the effects of hypoxia and carbohydrate feedings on recovery on muscle glycogen, specific mitochondrial genes related to exercise training, and oxidative stress.

CONFIDENTIALITY

1. Your records will be kept private and not be released without consent except as required by law.
2. Only the researcher and his research assistants will have access to the files; representatives of the U. S. Army Medical Research and Materiel Command (or the DOD) are authorized to review research records.
3. Your identity will be kept confidential.
4. If the results of this study are written in a scientific journal or presented at a scientific meeting, names will not be used.
5. All data, identified only by an ID #, will be stored in our laboratory.
6. The signed consent form and information sheet will be stored in a locked cabinet separate from the data.

COMPENSATION FOR INJURY

Although we believe that the risk of taking part in this study is minimal, the following liability statement is required in all University of Montana consent forms. *In the event that you are injured as a result of this research you should individually seek appropriate medical treatment. If the injury is caused by negligence of the University or any of its employees, you may be entitled to reimbursement pursuant to the Comprehensive State Insurance Plan established by the Department of Administration under the authority of M.C.A., Title2, Chapter 9. In the event of a claim for such injury, further information may be obtained from the University's Claim representative or University Legal Counsel.*

VOLUNTARY PARTICIPATION AND WITHDRAWAL

It is important that you realize that you are free to withdraw from the study at any time. As mentioned above, even if you decide to drop out of the study, you will receive full compensation for all the test sessions you complete or initiate. A copy of this consent form will be provided for you at your request. In addition, the data collected during this study will be done at no cost to you.

QUESTIONS

You may wish to discuss this with others before you agree to take part in this study. If you have any questions about the research now or during the study contact Dr. Brent C. Ruby, PhD at

Project funded by the Defense Medical Research and Development Program, a Department of Defense (DOD) organization

(406) 243-2117 (office) or (406) 396-4382. If you have any questions regarding your rights as a subject, you may contact the chair of the IRB through the University of Montana Research Office at (406) 243-6670.

STATEMENT OF CONSENT

I have read the above statements and understand the risks involved with this study. I authorize Dr. Brent C. Ruby, PhD and such assistants that he may designate, to administer and conduct the testing as safely as possible with a minimal amount of discomfort. If I have additional questions, I may contact Dr. Brent C. Ruby, PhD at home (406) 542-2513, cell (406) 396-4382 or at the Human Performance Laboratory (406) 243-2117.

Participant (print) _____

Signature _____

Date _____

Disclosure of Personal Health Information

My individual health information that may be used to conduct this research includes:

Age, height, weight, %body fat, VO_2 max, gene expression in response to exercise/hypoxia, muscle glycogen levels, and markers of oxidative stress.

I authorize *Dr. Brent C. Ruby, PhD* and the researcher's staff *and* representatives of the USAMRMC to use my individual health information for the purpose of conducting the research project entitled "The impact of hypoxia and carbohydrate feedings on recovery from exercise."

Since I receive compensation for participating in this study, identifying information about me may be used as necessary to provide compensation.

Signature: _____ Date: _____

STATEMENT OF CONSENT TO BE PHOTOGRAPHED DURING DATA COLLECTION

During the study, I understand that pictures may be taken. I provide my consent to having my picture taken during the course of the research study. I provide my consent that my picture may be used in some presentations related to this study. If pictures are used at any time for presentation, names and physiological data will not be associated with them.

Signature _____ Date _____

Project funded by the Defense Medical Research and Development Program, a Department of Defense (DOD) organization

Approval Expires On 8-28-2012
Date Approved By UM-IRB 8-29-2011
IRB-Chair

Appendix 2: HRPO Approval

Classification: UNCLASSIFIED
Caveats: NONE

SUBJECT: Initial Approval for Protocol, "The Impact of Hypoxia and Carbohydrate Feedings on Recovery from Exercise," Submitted by Brent C. Ruby, PhD, University of Montana, Missoula, Montana, in Support of the Proposal, "Evaluation of the Human/Extreme Environment Interaction: Implications for Enhancing Operational Performance and Recovery," Submitted by Brent C. Ruby, PhD, University of Montana, Missoula, Montana, Proposal Log Number DM090529, Award Number W81XWH-10-2-0120, HRPO Log Number A-16417.2

1. The subject protocol was approved by the University of Montana Institutional Review Board (IRB) on 29 August 2011. This protocol was reviewed by the U.S. Army Medical Research and Materiel Command (USAMRMC) Office of Research Protections (ORP), Human Research Protections Office (HRPO) and found to comply with applicable DOD, U.S. Army, and USAMRMC human subjects protection requirements.

2. This greater than minimal risk study is approved for the enrollment of 12 subjects.

3. Please note the following reporting obligations. Failure to comply could result in suspension of funding.

a. Major modifications to the research protocol and any modifications that could potentially increase risk to subjects must be submitted to the U. S. Army Medical Research and Materiel Command (USAMRMC) Office of Research Protections (ORP), Human Research Protections Office (HRPO) for approval prior to implementation. Major modifications include changes in study design, a change in Principal Investigator, change or addition of an institution, change in age range, change in/addition to the study population or a change that could potentially increase risks to subjects. All other amendments must be submitted with the continuing review report to the HRPO for acceptance.

b. All unanticipated problems involving risk to subjects or others, serious adverse events related to participation in the study and related a subject deaths must be promptly reported by phone (301-619-2165), by email (hrpo@amedd.army.mil), or by facsimile (301-619-7803) to the HRPO. A complete written report will follow the initial

notification. In addition to the methods above, the complete report can be sent to the U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RP, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

c. Suspensions, clinical holds (voluntary or involuntary), or terminations of this research by the IRB, the institution, the Sponsor, or regulatory agencies will be promptly reported to the USAMRMC ORP HRPO.

d. Any deviation to the protocol that may have an adverse effect on the safety or rights of the subject or the integrity of the study must be reported to the HRPO as soon as the deviation is identified.

e. A copy of the continuing review report and the re-approval notification by the University of Montana IRB must be submitted to the HRPO as soon as possible after receipt of approval. According to our records, it appears the current approval by the University of Montana IRB expires on 28 August 2012. Please note that the HRPO also conducts random audits at the time of continuing review and additional information and documentation may be requested at that time.

f. The final study report submitted to the University of Montana IRB, including a copy of any acknowledgement documentation and any supporting documents, must be submitted to the HRPO as soon as all documents become available.

g. The knowledge of any pending compliance inspection/visit by the FDA, OHRP, or other government agency concerning this research, the issuance of Inspection Reports, FDA Form 483, warning letters or actions taken by any regulatory agencies including legal or medical actions and any instances of serious or continuing noncompliance with the regulations or requirements must be reported immediately to the HRPO.

4. Please Note: The U S Army Medical Research and Materiel Command, Office of Research Protections, Human Research Protections Office conducts random site visits as part of its responsibility for compliance oversight. Accurate and complete study records must be maintained and made available to representatives of the U.S. Army Medical Research and Materiel Command as a part of their responsibility to protect human subjects in research. Research records must be stored in a confidential manner so as to protect the confidentiality of subject information.

5. Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grants Officer can authorize expenditure of funds. It is recommended that you contact the appropriate contract specialist or contracting

officer regarding the expenditure of funds for your project.

6. The HRPO point of contact for this study is Sharon A. Evans, PhD, CIP, Human Subjects Protection Scientist, at 301-619-2256/sharon.a.evans.ctr@us.army.mil.

LAURA RUSE BROSCH, RN, PhD
Director, Office of Research Protections
Human Research Protection Office
U.S. Army Medical Research and Materiel Command

Note: The official copy of this approval memo is housed with the protocol file at the Office of Research Protections, Human Research Protections Office, 504 Scott Street, Fort Detrick, MD 21702. Signed copies will be provided upon request.
Classification: UNCLASSIFIED
Caveats: NONE